

ORIGINAL ARTICLE

Platelet alpha granules in BLOC-2 and BLOC-3 subtypes of Hermansky-Pudlak syndrome

MARJAN HUIZING¹, JENNIFER M. PARKES¹, AMANDA HELIP-WOOLEY¹,
JAMES G. WHITE², & WILLIAM A. GAHL^{1,3}

¹National Human Genome Research Institute, National Institutes of Health, Section on Human Biochemical Genetics, Medical Genetics Branch, Bethesda, MD, USA, ²Department of Laboratory Medicine, University of Minnesota, Minneapolis, MN, USA, and ³National Institutes of Health, Office of Rare Diseases, Intramural Program, Bethesda, MD, USA

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Abstract

Hermansky-Pudlak syndrome (HPS) is a disorder of lysosome-related organelle biogenesis that displays genetic locus heterogeneity. The eight known HPS proteins combine in functional complexes, two of which are called BLOC-2 and BLOC-3; a BLOC is a Biogenesis of Lysosome-related Organelles Complex. Organelles affected in HPS include the melanosome, resulting in hypopigmentation, and the platelet delta (dense) granule, resulting in prolonged bleeding times. Whole mount electron microscopy (EM) detects the absence of platelet delta granules and confirms the diagnosis of HPS. To date, the status of other organelles and granules in HPS platelets has not been documented. We performed ultrastructural studies on platelets of patients with different genetic forms of HPS, specifically those comprising the BLOC-2 and BLOC-3 subtypes. No differences in distribution, size or quantity of other platelet organelles and membrane structures could be detected in our patients. Since alpha and delta granules are formed from multivesicular bodies in the megakaryocyte, and since only delta granules are defective in HPS, we conclude that HPS genes function within the portion of delta granule biogenesis that has diverged from that of alpha granules. Thus, it is unlikely that the generalized bleeding diathesis of HPS is attributed to a deficiency of alpha granules.

Keywords: *Hermansky-Pudlak syndrome, delta granule, alpha granule, BLOC, lysosome-related organelle*

Introduction

Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disorder characterized by oculocutaneous albinism and platelet storage pool deficiency [1–4]. HPS occurs rarely in the general population, but one genetic subtype has a frequency of 1 in 1800 in northwest Puerto Rico due to a founder mutation [5]. In HPS, oculocutaneous albinism results from an inability to place a normal contingent of pigmented melanosomes in the dendrites of melanocytes; this appears to be caused by mistrafficking of melanogenic proteins to melanosomes [4, 6, 7]. Individuals with HPS also present with a bleeding diathesis due to an absence of delta granules within platelets [2, 8]. Some patients exhibit granulomatous colitis or a fatal pulmonary fibrosis, and all Puerto Rican HPS-1

patients manifest intracellular ceroid lipofuscin, an autofluorescent, lipid-protein complex [2, 9].

Eight genes are known to cause distinct HPS subtypes in humans (HPS-1 through HPS-8) [10]. Except for HPS-2, which results from a defect in the β 3A subunit of adaptor complex-3 (AP3), all human subtypes of HPS are caused by mutations in genes coding for proteins with unknown functions [4, 10]. These HPS proteins interact with each other in Biogenesis of Lysosome-related Organelle Complexes, or BLOCs. HPS7 and HPS8 are found in BLOC-1; HPS3, HPS5, and HPS6 interact in BLOC-2; and HPS1 and HPS4 are components of BLOC-3 [10–13]. Mouse models exist for all the human HPS subtypes and for other disorders combining hypopigmentation and bleeding [3, 10, 14]. The HPS genes are thought to be involved in the

biogenesis of melanosomes and platelet delta granules, accounting for hypopigmentation of hair, skin, and eyes and extended bleeding times, respectively [3, 4, 10, 13]. Normal platelets contain three to eight delta granules filled with serotonin, pyrophosphate, calcium, ATP, and ADP, which stimulate platelet aggregation [15–19]. The absence of platelet delta granules, demonstrated on whole-mount electron microscopy, is utilized for the definitive diagnosis of HPS [8].

Compared with delta granules, alpha granules are more abundant within platelets, numbering 50 to 80 per platelet. Alpha granules contain a variety of proteins involved in adhesion and repair mechanisms such as proteoglycans, protease inhibitors, adhesive glycoproteins, and haemostasis factors [18, 20]. Platelets also contain a variety of other specialized organelles, vesicular systems and particles including lysosomes, mitochondria, peroxisomes, glycogen granules, and microtubules [15–18]. Platelets have a complex network of membranes that consist of an open canalicular system, connecting the cytoplasm with the surrounding medium, and the dense tubular system, containing important metabolic enzymes [15–18].

Although the absence of delta granules in HPS platelets has long been considered to be an isolated deficiency, this has never been formally shown, particularly in the different genetic subtypes of the disorder. We now demonstrate normal alpha-granule numbers, distribution, and morphology, as well as a normal contingent of other intracellular organelles, in the platelets of HPS patients with BLOC-2 and BLOC-3 defects.

Methods

Patients and cells

All patients were enrolled in a protocol approved by the National Institute of Child Health and Human Development and the National Human Genome Research Institutional Review Boards to study the clinical and molecular features of HPS. Written informed consent was acquired from the patient or the patient's parent. Patient numbers correspond to a master file of all NIH patients with HPS. The diagnosis of HPS was based on the presence of oculocutaneous albinism and the absence of platelet delta granules on whole-mount electron microscopy. Subtyping of the patients was performed by mutation analysis of the human HPS genes [21–24].

Preparation of blood and platelets

Patient blood was mixed with citric acid dextrose (CCD) in a ratio of nine parts blood to one part anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation at $100 \times g$ for

20 min at room temperature. Platelet counts and volume were determined using a Coulter Heme X system. When necessary, platelet counts were adjusted to $300,000/\text{mm}^3$.

Whole mount electron microscopy of platelet delta granules

Small drops of citrate PRP were placed on formvar-coated, carbon-stabilized grids and rinsed with drops of sterilized water, dried from the edges with filter paper, and air-dried to remove residual moisture [8]. The grids were examined without fixation or staining in a Philips (F.E.I. Co., Hillsboro, OR, USA) 301 electron microscope.

Transmission electron microscopy of platelets

Fixation of control and patient citrate PRP was performed by adding an equal volume of 0.1% glutaraldehyde in White's saline [25]. After 15 min the samples were centrifuged to pellets, the supernatant fixative removed, replaced with 3% glutaraldehyde in the same buffer, incubated at 4°C for 30 min and sedimented to pellets. Supernatants were removed and replaced with either 1% osmic acid in Zetterquist's buffer or 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for 1 h at 4°C . All samples were dehydrated in a graded series of alcohol and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate to enhance contrast. Examination was carried out in a Philips (F.E.I. Co.) 301 electron microscope.

Results

Clinical findings in patients

Patient #139-1 was a 43-year-old Caucasian male recently diagnosed with HPS based upon the absence of platelet delta granules. Sequencing revealed a homozygous c.1189delC (p.Q397delC) mutation in exon 13 of the *HPS1* gene (GenBank NM_000195). The patient was born with oculocutaneous albinism, nystagmus, and decreased visual acuity. He had experienced bruising, epistaxis, and prolonged bleeding with minor cuts, but never required a transfusion. Tooth extraction and a tonsillectomy were performed in childhood without major complications. The patient reported dyspnea on walking one mile or two flights of stairs. He had no history of neurological or gastrointestinal complications, but had seborrheic keratoses and at age 40 developed a staphylococcal infection following arthroscopic debridement of his right knee joint.

On examination, height was 186 cm and weight 99.5 kg. The hair colour was white. The iris was slate

gray and the fundus appeared normal. Visual acuity was 20/120 od and 20/100 os. An echocardiogram showed mild dilatation of the right atrium and ventricle, mild pulmonary hypertension, and a mildly dilated aortic root and ascending thoracic aorta. Chest CT scan showed mild to moderate interstitial lung disease. A CAT scan of the cerebrum appeared normal. Pulmonary function tests revealed an FVC 94% of predicted, FEV₁ 87% of predicted, TLC 99% of predicted, and DL_{CO} 73% of predicted.

Patient #143-1 was a 39-year-old Puerto Rican male diagnosed with HPS-1 in his teens. Mutation analysis confirmed homozygosity for the Puerto Rican founder mutation c.1472_1487 dup16 in exon 15 of *HPS1*. The patient had undergone a lung transplantation at age 38 due to progressive pulmonary fibrosis [26]. He also had elbow surgery secondary to bony overgrowth at 18 years of age and repair of torn cartilage in his right knee at age 27.

On examination, height was 169.5 cm and weight was 90.5 kg. Visual acuity was 20/250 in both eyes and nystagmus was present. Solar keratoses with multiple hyperpigmented areas were present on the arms and face, with scattered actinic keratoses. The hair was white, and iris transillumination was present bilaterally. An electrocardiogram showed sinus rhythm with a rate of 80 and multiple atrial premature complexes. Pulmonary function tests showed a mild restrictive defect with forced vital capacity 70% of predicted. Computerized tomography of the head was normal.

Patient #91-3 was a 7-year-old male of German and Swiss ancestry whose clinical summary was previously reported [23]. Briefly, he had a history of epistaxis and bruising with a visual acuity of 20/160 od and 20/200 os. The hair and skin were mildly hypopigmented. Mutation analysis revealed two compound heterozygous mutations in *HPS3* (GenBank NM_032383), i.e., c.C1329T (p.R397W, exon 6) and IVS14 + 1G > C [23].

Patient #37-4 was a 19-year-old female from the subcontinent of India whose clinical summary was previously reported [22]. Visual acuity was 20/125 od and 20/200 os. The patient had a bleeding diathesis typical for HPS, but no colitis on colonoscopy. The FVC was 57% of predicted. Mutation analysis revealed two compound heterozygous mutations in *HPS4* (GenBank NM_022081), i.e., c.412 G > T (p.E138X, exon 6) and c.664 G > T (p.E222X, exon 8) [22].

Patient #153-4 was a 2-year-old male, and the brother of HPS patients #30 and #38, which were previously described [22]. Oculocutaneous albinism was apparent at birth. At one month of age, a perirectal abscess required surgical drainage and at 6 weeks of age the infant developed pertussis and was hospitalized for 3 days. At 10 weeks of age, he was hospitalized for an infection with respiratory

syncytial virus. He had easy bruising but no nosebleeds or blood in the stool.

On examination, height was 87.5 cm and weight was 13.1 kg. The hair was fine and white-blond and his skin was fair. The irides were blue, and the fundus was blond. Right beating nystagmus was observed and significant iris transillumination was apparent bilaterally. He had no respiratory distress and his heart rate and rhythm were normal. Mutation analysis verified compound heterozygous *HPS4* mutations, i.e., c.461A > G (p.H154R, exon 6) and c.649C > T (p.R217X, exon 8).

Patient #48-5 was a 51-year-old woman of Swiss extraction diagnosed at age 42 based upon absence of platelet dense bodies. Her clinical history was previously described [24]. Her visual acuity was 20/125 od and 20/100 os. The FVC was 100% of predicted, the FEV₁ 115% of predicted, the TLC 100% of predicted, and the DL_{CO} 123% of predicted. She had a history of cholelithiasis and cholecystectomy, appendectomy, and strabismus repairs. This patient had two compound heterozygous mutations in *HPS5* (GenBank NM_181507), i.e., c.1871 T > G (p.L624R, exon 16) and c.3293 C > T (p.T1098I, exon 22) [24].

Ultrastructural analysis of platelet granules

The HPS patients included in this study bear mutations in genes that produce BLOC-2 proteins (patients #48-5 and #91-3) or BLOC-3 proteins (patients #37-4, #139-1, #143-1, and #153-4). Whole mount EM showed absence of platelet delta granules, a hallmark of HPS, in all cases; Figure 1 shows representative images of BLOC-2 (Figure 1B) and BLOC-3 (Figure 1C) defects. Normal delta granules appear electron-dense on a whole mount electron micrograph (Figure 1A, arrows).

To study the alpha granule status of our patient group, platelet rich plasma was collected and fixed for transmission EM. Figure 2 presents representative micrographs containing several platelets of each patient. The platelets have either a discoid/round or a spiny elongated spherical appearance, depending upon the cutting angle of the thin section and the activation state of the platelets at the time of fixation. The numbers and shapes of whole platelets in HPS patients appear similar to those of controls (Figure 2). Apart from the absence of delta granules (*dg* in Figure 2A), no gross histological granule or membrane abnormalities were identified in the HPS platelets (Figure 2B–H) compared to control platelets (Figure 2A). The number of alpha granules (*ag*) present in each plane of sectioning was ~8–20 per platelet for both patients and controls. The distribution of other identifiable platelet components such as mitochondria, glycogen particles and membrane structures (indicated by arrows in each image) also appeared normal in HPS patients.

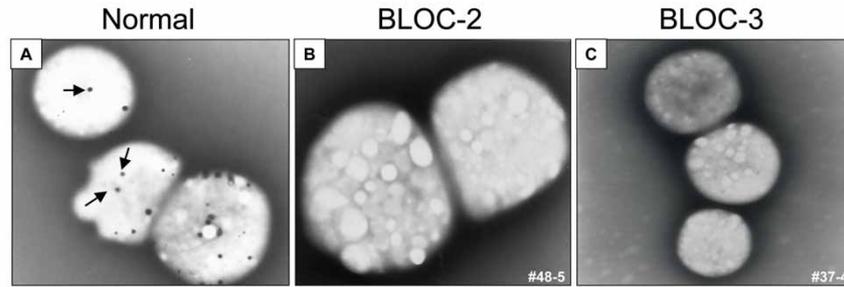


Figure 1. Whole mount EM prepared from platelet rich plasma. (A) Normal control platelets each contain several dense granules (arrows) ($\times 4000$). (B) Patient #48-5 platelets (representing a BLOC-2 defect), devoid of dense granules ($\times 6000$). (C) Patient #37-4 platelets (representing a BLOC-3 defect), lacking dense granules ($\times 6000$).

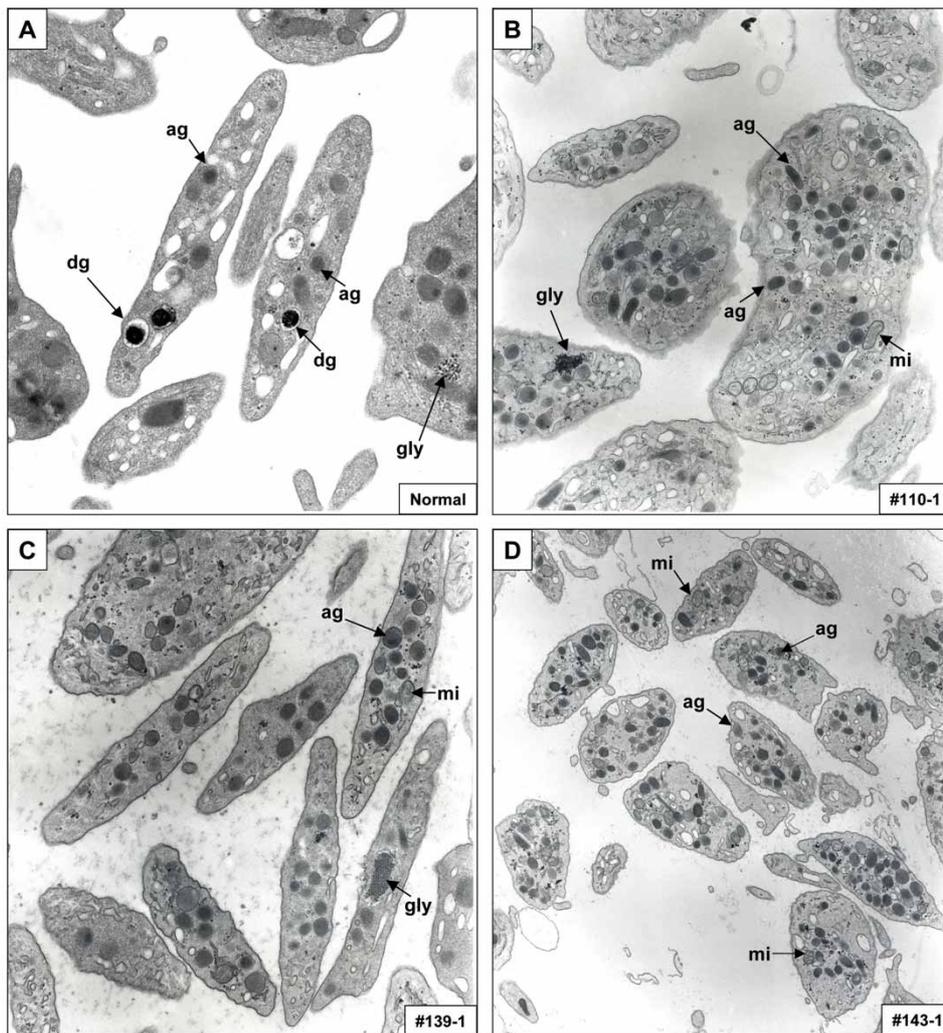


Figure 2. Thin sections of normal platelets and platelets from HPS patients. (A) Control platelets ($\times 16000$); (B) Patient #110-1 ($\times 14000$); (C) Patient #139-1 ($\times 14000$); (D) Patient #143-1 (original magnification $\times 11000$); (E) Patient #37-4 ($\times 20000$); (F) Patient #153-4 ($\times 20000$); (G) Patient #91-3 ($\times 22000$); (H) Patient #48-5 ($\times 12000$). The HPS patients represent defects in BLOC-3 (B-F) or BLOC-2 (G,H). Normal platelets (A) contain dense granules (*dg*), which are lacking in all HPS patients. Other cytoplasmic components include alpha granules (*ag*), mitochondria (*mi*), glycogen particles (*gly*), and several other vesicular/vacuolar and membrane structures [18].

Other structures that are difficult to classify are visible on the micrographs. This group of structures includes peroxisomes, lysosomes, and membranes of the open canalicular system and dense

tubular system. We did not see abnormal numbers, shapes or distributions of these structures or membranes in HPS patients compared to controls.

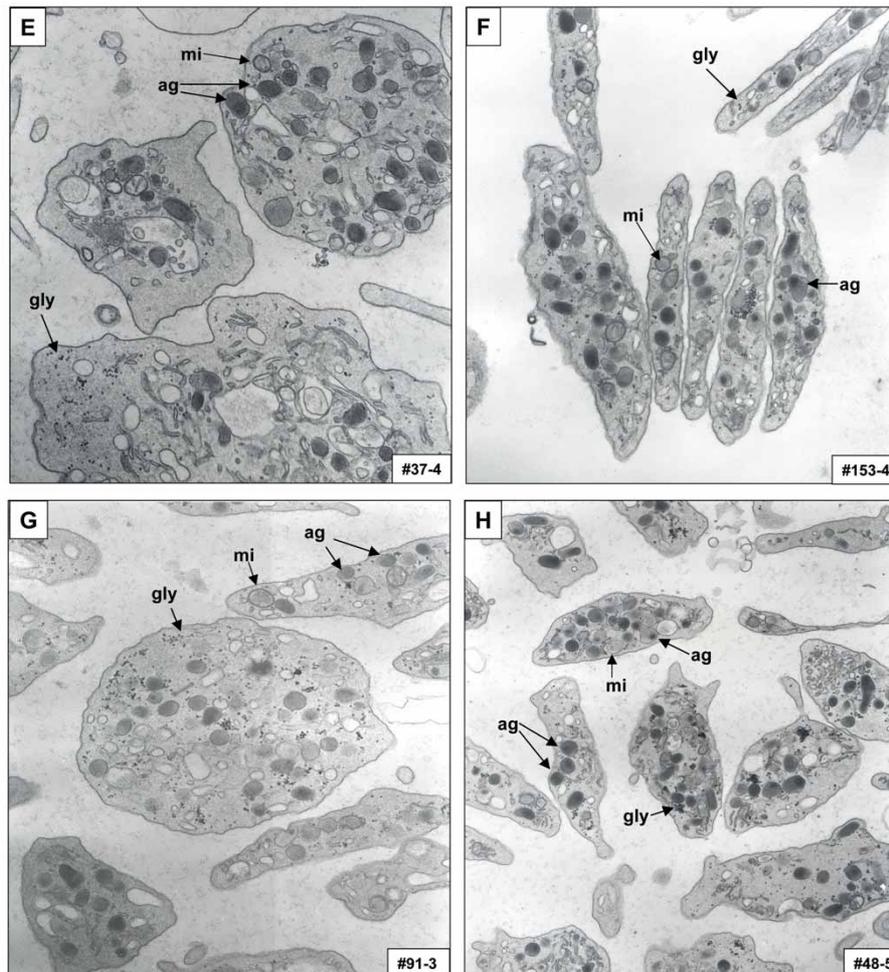


Figure 2. Continued.

Figure 3 shows a high magnification view of a control platelet (Figure 3A), a platelet of patient #48-5 representing a BLOC-2 defect (Figure 3B), and a platelet of patient #153-4 representing a BLOC-3 defect (Figure 3C). Detailed structures are indicated by arrows in each of the images. First, a circumferential coil of microtubules (*mt*) can be recognized just under the platelet membrane; these microtubules assist the platelets in maintaining their shape. Second, delta granules (*dg*) are seen only in the control platelet (Figure 3A), and are characterized by the presence of an intensely electron dense core surrounded by a clear space and a single membrane. The dense material may or may not completely fill the lumen of the granule [18]. Delta granules are not seen in any of the HPS patients' platelets, as expected. Thin sectioning of control platelets can also yield cutting planes that do not include delta granules, and therefore thin sectioning followed by transmission EM is not always reliable for diagnosing delta granule deficiencies. Whole mount EM (Figure 1) is a more reliable method for this purpose.

Third, numerous alpha granules (*ag*) can be recognized in each platelet. Alpha granules appear as spherical or ovoid organelles surrounded by a single membrane and containing an electron-lucent gray matrix and often a darker round core filled with proteoglycans [18]. Figure 3 and additional higher magnification studies (not shown) revealed no apparent differences in alpha granule architecture, distribution, or numbers in HPS platelets compared to controls.

Finally, many other cytoplasmic organelles and membrane structures are visible on each micrograph. These include mitochondria (*mi*), glycogen particles (*gly*), lysosomes, peroxisomes, and multiple membrane systems, including the open canalicular system and dense tubular system. No abnormalities in these structures or membranes were apparent in HPS platelets.

Discussion

Platelets are anucleated cells rich in specialized storage granules that release their contents during hemostasis [16–20]. In addition to complex

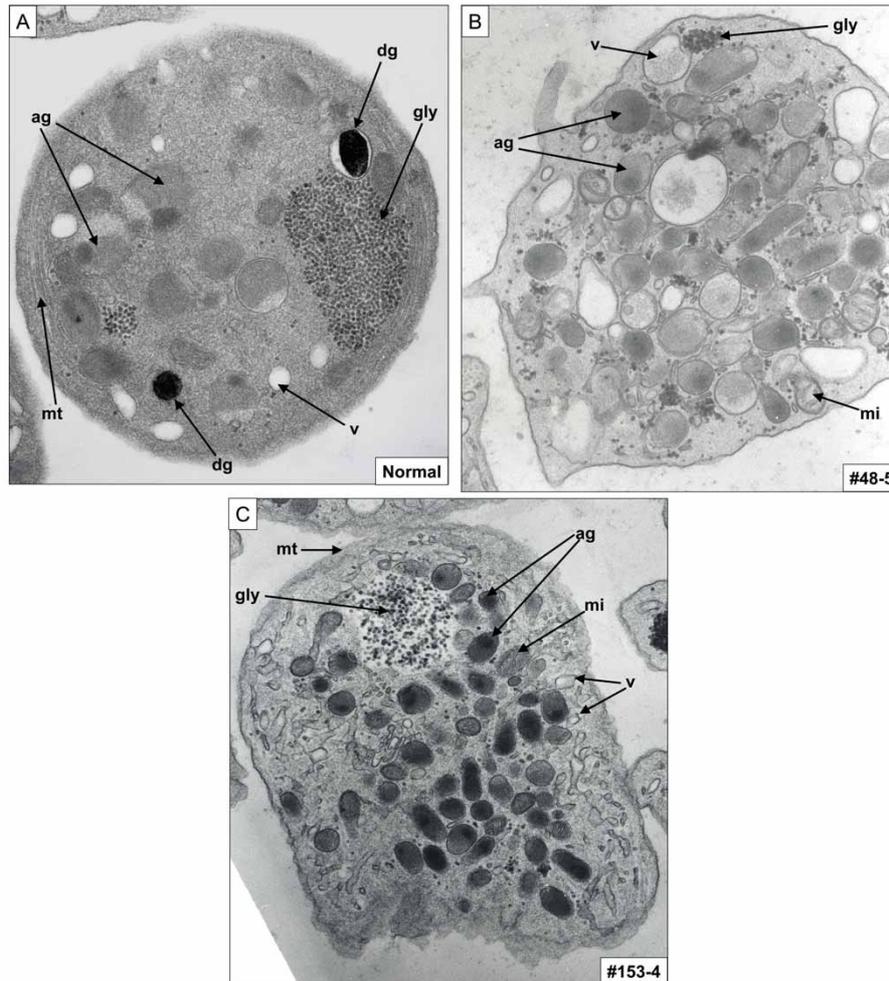


Figure 3. Thin section of one platelet from a normal individual, a BLOC-2 patient, and a BLOC-3 patient. (A) Normal platelet ($\times 36000$). (B) Platelet of #48-5, representing a BLOC-2 defect ($\times 36000$). (C) Platelet of #153-4, representing a BLOC-3 defect ($\times 28000$). The cytoplasm of each platelet is filled with alpha granules (*ag*), recognized by their homogeneous electron density with a denser core, mitochondria (*mi*), a coil of circumferential microtubules (*mt*) that support the platelet's shape, glycogen particles (*gly*), and various other vesicular/vacuolar/membrane structures (*v*) including peroxisomes, lysosomes and membranes of the open canalicular system and the dense tubular system (see [18]).

canalicular and tubular membrane systems, mitochondria and glycogen particles, two ultrastructurally distinct granule populations, delta granules and alpha granules, are recognized in platelets. Delta granules are 200–300 nm electron-dense structures containing small non-protein molecules such as serotonin, ADP, ATP, pyrophosphate, and calcium [15, 17, 19]. Alpha granules are 200–500 nm organelles containing various large proteins (e.g., fibronectin, thrombospondin, vonWillebrand Factor, proteoglycans, albumin, immunoglobulins) that function in hemostasis, inflammation, wound healing and cell-matrix interactions [18, 20].

The formation and packaging of platelet granules remains poorly understood. Several human and murine genes have been linked to delta granule biogenesis; some encode known vesicle trafficking proteins whereas others encode components of BLOC protein complexes, whose exact functions are unknown [4, 10, 13, 14, 19]. Similarly, little is known about the biogenesis of alpha granules,

although a few genes (e.g., *GATA1*, *FLI1*, *NFE2*) [27–29], glycoprotein Ib-beta [30] and *VPS33B* [31]) have been implicated in this process. A mutation in *RABGGTA*, coding for subunit A of rab-geranylgeranyl transferase [32], causes deficiency of both alpha and delta granules in the *gunmetal* mouse.

There are several reasons to question whether multiple intracellular organelles might be affected in HPS, long considered to involve an isolated deficiency of delta granules. First, alpha and delta granules contain common membrane markers, including P-selectin, CD63 and Rab27B [33]. Second, a combined deficiency of alpha and delta granules exists in the *gunmetal* mouse [32] and human alpha-delta storage pool deficiency [19]. Third, certain HPS patients display defects of von Willebrand Factor, an alpha granule protein [34]. Finally, some evidence suggests that both alpha and delta granules develop from the same multivesicular bodies in megakaryocytes [35–37]. The mechanism

by which these granules develop into distinct entities is not clear, but that mechanism might explain the apparent inverse relationship between the number of alpha and delta granules seen in ARC syndrome (arthrogryposis multiplex congenital, renal dysfunction, and cholestasis). In this disorder due to mutations in *VPS33B*, alpha granule deficiency is associated with an increased number of delta granules [31]. Delta granules could be the default destiny for multivesicular body membranes not used for alpha granule formation. Alternatively, deficiency of alpha-granules within megakaryocytes could trigger the transport of additional delta granules into proplatelets.

Based upon these recent findings, we investigated the ultrastructural features, numbers and distribution of alpha granules in HPS platelets. Our studies did not identify any ultrastructural defect in alpha granules of BLOC-2 or BLOC-3 deficient platelets. Moreover, other vesicular and membrane structures in platelets, including mitochondria, peroxisomes, lysosomes, glycogen particles, and several membrane structures also appeared to be unaffected by the HPS mutations.

While the ultrastructural morphology and number of alpha granules appears to be normal in BLOC-2 or BLOC-3 deficient platelets, we cannot definitively rule out the mistrafficking of individual proteins or other alpha granule-specific contents. Other techniques besides transmission EM, such as immuno-EM, specialized staining procedures, and proteomic analyses, would be helpful adjunctive methods. In future studies, the lysosome might be of interest as a target of investigation, since its formation may follow a pathway similar to that of the delta granule [38]. Platelet lysosomes measure between 175 and 200 nm and contain primarily acid proteases (cathepsins, carboxypeptidases) and hydrolases (heparinase, glucosidase, fucosidase) [18, 38].

Conclusion

The mechanism of alpha granule formation may share early elements with the mechanism of delta granule formation. However, the BLOC-2 and BLOC-3 proteins of HPS apparently operate within aspects of platelet vesicle formation that are specific to delta granule formation, i.e., after the divergence of the two pathways. We conclude that alpha granule deficits do not contribute to the bleeding diathesis of HPS patients with BLOC-2 or BLOC-3 subtypes, i.e., HPS-3/HPS-5/HPS-6 or HPS-1/HPS-4.

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